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The modulation of cell surface cAMP receptors from *Dictyostelium discoideum* by ammonium sulfate

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Dictyostelium discoideum cells contain a heterogeneous population of cell surface cAMP receptors with components possessing different affinities (K_d between 15 and 450 nM) and different off-rates of the cAMP-receptor complex ($t_{1/2}$ between 0.7 and 150 s). The association of cAMP to the receptor and the dissociation of the cAMP-receptor complex still occur in the presence of 3.4 M ammonium sulfate. However, these processes are strongly altered. (1) Low concentrations of ammonium sulfate (≈ 50 mM) induce an approx. 2-fold increase of the number of cAMP binding sites. The same effect is induced by millimolar concentrations of CaCl_2 . Ammonium sulfate and CaCl_2 are not additive, which suggests that these salts may act via the same mechanism. (2) High concentrations of ammonium sulfate (3.4 M) induce an alteration in the proportioning of the various cAMP binding sites to the components with the highest affinity. (3) High concentrations of ammonium sulfate (3.4 M) retard the dissociation of all binding sites about 3–6-fold, thus giving rise to an increase in the affinity of all cAMP-binding components.

Introduction

cAMP has in *Dictyostelium* an extracellular function as a chemoattractant during cell aggregation [1] and morphogenesis [2]. This nucleotide is detected by highly specific cell surface receptors [3] which transduce the signal to different effectors, such as adenylate cyclase, guanylate cyclase, and calcium (for review see Ref. 4). These cell surface cAMP receptors are heterogeneous in respect to their kinetic properties (Ref. 5 and unpublished observations). At least four components can be distinguished which have different affinities (apparent K_d between 15 and 450 nM) and different dissociation rates of the cAMP-receptor complex ($t_{1/2}$ between 0.7 and 150 s, at 20°C).

Recently we have introduced the ammonium sulfate stabilization assay to measure cAMP binding to *D. discoideum* cells [3]. In this assay

[^3H]cAMP is incubated with the cells at 0°C for 1 min, which is followed by a 12-fold dilution of the incubation mixture with 3.4 M ammonium sulfate (final concentration). Preliminary experiments suggested that ammonium sulfate prevented the dissociation of the cAMP-receptor complex, and the new association of cAMP to free receptors. This ‘freezing’ of the binding equilibrium by ammonium sulfate in *D. discoideum* would be very similar to the effect of ammonium sulfate on other receptors [6,7]. More detailed experiments revealed that in *D. discoideum* association and dissociation still take place in the presence of ammonium sulfate.

In the present report it is shown that ammonium sulfate has a multiple effect on cell surface cAMP binding sites in *D. discoideum*. (1) The number of binding sites is increased about 2-fold. (2) The ratio of the different cAMP-binding sites

is shifted to the components with the higher affinities. (3) The rate of dissociation of all components is reduced 3–6-fold.

Materials and Methods

Materials. [2,8- ^3H]cAMP (1.5 TBq/mmol) was purchased from the Radiochemical Centre (Buckinghamshire, U.K.), cAMP was from Boehringer (Mannheim, F.R.G.), caffeine was from the British Drug House (Poole, U.K.), and dithiothreitol was from Sigma Chemical Co. (St. Louis, U.S.A.).

Culture conditions. *D. discoideum*, NC4(H), was grown in association with *Escherichia coli*, 281, on a solid medium containing 3.3 g peptone, 3.3 g glucose, 4.5 g KH_2PO_4 , 1.5 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ and 15 g agar per liter. Cells were harvested in the late log phase with buffer 1 (10 mM sodium/potassium phosphate buffer, pH 6.5) and freed from bacteria by repeated centrifugations at $100 \times g$ for 4 min. Cells were starved in buffer 1 by shaking for 5 h at density of 10^7 cells/ml.

cAMP-binding assays. cAMP was measured at 0°C in assay buffer (buffer 1 with 5 mM dithiothreitol, an inhibitor of phosphodiesterase in *D. discoideum* [8]) by the pellet assay [9], or by centrifugation of the cells through silicon oil [10]. In the pellet assay the incubation mixture (0.1 or 1.0 ml) contained assay buffer, different concentrations [^3H]cAMP and cAMP, different concentrations of ammonium sulfate and 10^7 cells. After the desired incubation period the samples were centrifuged for 2 min at $10\,000 \times g$. The supernatant was removed, the walls of the tubes were dried with tissue paper, and the pellet was dissolved in 0.1 ml 1 M acetic acid. 1.3 ml scintillation liquid (Instagel, Packard) was added to the test tubes; the tubes were placed in a 20-ml counting vial, and the radioactivity was determined in a Rack β liquid scintillation counter (LKB). In the experiments with ammonium sulfate 0.5 mg bovine serum albumin was included in the incubation mixture; during centrifugation cells precipitate faster than the denatured bovine serum albumin, which improves the stability of the otherwise loose cell pellet.

The incubation mixture for the silicon oil centrifugation assay contained assay buffer, differ-

ent concentrations of [^3H]cAMP and cAMP and 10^7 cells in a total volume of 100 μl . After the desired incubation period 95 μl of the incubation mixture were centrifuged through 200 μl silicon oil (AR20/AR200 = 11/4). The tip of the tubes containing the cell pellet was cut and placed in a 5 ml picovial. The radioactivity was determined in 1.5 ml Instagel.

The binding data were corrected for quenching of the [^3H]cAMP. This was measured for the different assays as follows. The binding assays were carried out in the absence of [^3H]cAMP as described above. For the pellet assay 10 μl [^3H]cAMP (about 50 000 cpm) were added to the dissolved cell pellet and to a tube containing 100 μl 1 M acetic acid. Instagel was added to both tubes and radioactivity was determined. For the silicon oil assay the cut tip containing the cell pellet was placed in the picovial and 10 μl [^3H]cAMP and 1.5 ml Instagel were added; the radioactivity was compared with that of 10 μl [^3H]cAMP placed directly in Instagel. The recovery was 93% for the pellet assay without ammonium sulfate, 95% for the pellet assay with 3.4 M ammonium sulfate, and 71% for the silicon oil centrifugation assay.

Nonspecific binding was determined by including 0.1 mM cAMP in the incubation mixture (or the preincubation mixture in Fig. 3). Nonspecific binding was 0.2% of the input radioactivity for the pellet assay in buffer, 1.3% for the pellet assay in 3.4 M ammonium sulfate, and 0.5% for the silicon oil centrifugation assay. Nonspecific binding has been subtracted from all data shown. The experiments were done at least three times, yielding similar results.

Results

Equilibrium kinetics

The binding of 1 nM [^3H]cAMP to *D. discoideum* cells at different ammonium sulfate concentrations reveals a multiphasic effect of the salt (Fig. 1). Low ammonium sulfate concentrations (up to 50 mM) induced an approx. 2-fold increase in cAMP binding. A further 4-fold increase in cAMP binding is observed between 50 mM and 1.5 M ammonium sulfate. Finally, at concentrations above 1.5 M an additional 4-fold increase

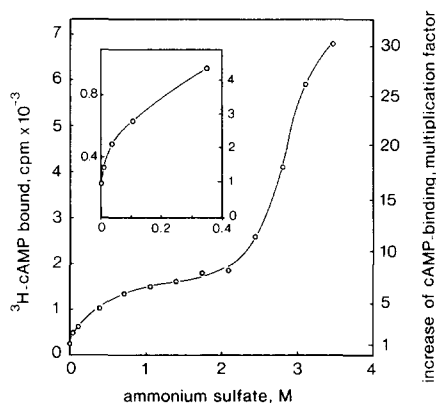


Fig. 1. Effect of ammonium sulfate on cAMP-binding. The incubation mixture contained in 1 ml assay buffer 1 nM [^3H]cAMP (36000 cpm), 0.5 mg bovine serum albumin, 10^7 cells, and different concentrations of ammonium sulfate. Samples were centrifuged after an incubation period of 5 min, and the radioactivity in the cell pellet was determined. Nonspecific binding was measured at each ammonium sulfate concentration and increased from 51 cpm in the absence to 365 cpm in the presence of 3.5 M ammonium sulfate. Inset: enlargement of the same data.

takes place. At the highest ammonium sulfate concentration tested (3.5 M, 87% saturated) specific binding is increased 30-fold if compared to the incubations without ammonium sulfate.

This experiment was performed at low cAMP concentrations. In order to determine the effect of ammonium sulfate on the affinity and the number of cAMP-binding sites, Scatchard plots were made at 0, 50 mM, 1.4 M and 3.4 M ammonium sulfate (Fig. 2). This indicates that the increase in cAMP-binding at low ammonium sulfate concentrations is due to an increase in the number of cAMP-binding sites, while the further increases of the binding of 1 nM [^3H]cAMP are due to increases in the apparent affinity of the cAMP-binding sites.

Association of cAMP to *D. discoideum* cells

The association of [^3H]cAMP to the cells was investigated by experiments shown in fig. 3. Cells were incubated in 1.0 ml with 1 nM [^3H]cAMP in the presence of 3.4 M ammonium sulfate (filled circles). Specific binding increases to an apparent equilibrium value obtained at about 5 min; half-maximal binding is obtained after about 30 s. Cells were preincubated with 10 nM [^3H]cAMP in

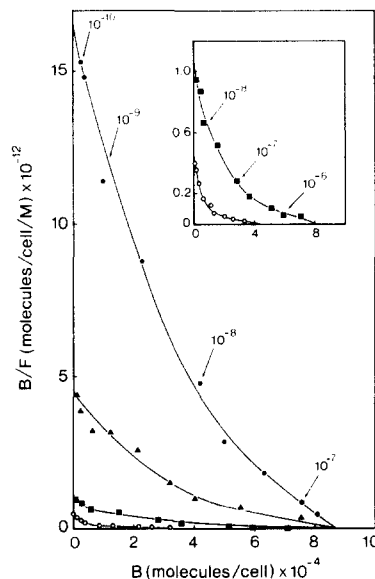


Fig. 2. Scatchard plot of cAMP-binding at different ammonium sulfate concentrations. The incubation mixture contained in 1 ml assay buffer, 10^7 cells, 0.5 mg bovine serum albumin, different cAMP concentrations in the absence (○) or presence of 50 nM (■), 1.4 M (▲) and 3.4 M (●) ammonium sulfate. Inset: enlargement of the same data.

0.1 ml for 1 min, followed by the addition of 0.9 ml ammonium sulfate (3.4 M final concentration, open circles). Again [^3H]cAMP binding increases to approximately the same equilibrium value as without preincubation. Clearly, association of [^3H]cAMP to *D. discoideum* cells can take place in the presence of 3.4 M ammonium sulfate, which is in contradiction with what we have reported previously [3]. In this figure a third experiment is shown (filled triangles) in which 3.4 M ammonium sulfate with 10^{-4} M cAMP is added after the 1 min preincubation period. Approx. 50–60% of the specific binding is released within 1 min. The remaining specific binding is released very slowly, if at all; after 60 min still about 30% of the [^3H]cAMP is bound to the cells. The association of 1 nM [^3H]cAMP to cells in buffer is shown in Fig. 3B. Association is very fast; half-maximal binding is obtained after about 3 s, which is about 10-fold faster than in the presence of 3.4 M ammonium sulfate.

Dissociation of the cAMP receptor complex

The dissociation of [^3H]cAMP from cells is

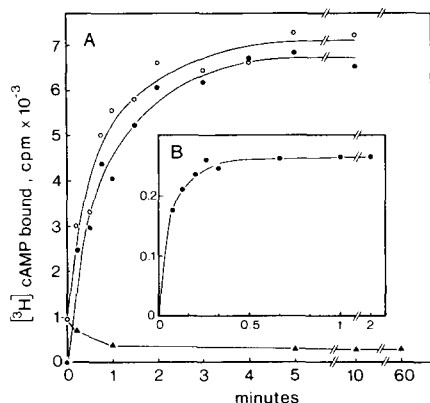


Fig. 3. The kinetics of association of cAMP to *D. discoideum* cells. A. Association in ammonium sulfate. Filled circles (●); the incubation mixture contained, in 1 ml assay buffer, 3.4 M ammonium sulfate, 1 nM [^3H]cAMP, 0.5 mg bovine serum albumin and 10^7 cells. Samples were centrifuged at the indicated times, and the radioactivity in the cell pellet was determined. Open circles (○); 10^7 cells were preincubated in 0.1 ml with assay buffer and 10 nM [^3H]cAMP for 1 min. At $t = 0$ min 850 μl 4 M ammonium sulfate and 50 μl bovine serum albumin (0.5 mg) were added. Samples were centrifuged at the indicated times. One sample was centrifuged before the addition of ammonium sulfate and represents the cAMP-binding at $t = 0$ min. Filled triangles (▲); same experiment as in (○), except that 850 μl 4 M ammonium sulfate with 0.1 mM cAMP was added. B. Association in buffer. The incubation mixture contained in 0.1 ml assay buffer 1 nM [^3H]cAMP, and 10^7 cells. At the times indicated 95 μl of the incubation mixture were centrifuged through silicon oil. Nonspecific binding was measured by including 0.1 mM cAMP in the incubation and preincubation mixtures and was determined at 0 min (○), and at 1 min (●, ○, ▲).

complex; previously we have reported two components with half-lives of dissociation at 20°C of about 1 s and 15 s, respectively [5]. Here we introduce a third component with a very slow off-rate ($t_{1/2} = 150$ s at 20°C). This component represents about 10% of the binding at 2 nM [^3H]cAMP, and only about 1–2% of the number of binding sites belong to this class (unpublished observation). These figures are very small and depend highly on the accuracy of the determination of nonspecific binding. Nonspecific binding is defined as the cell-associated radioactivity not bound to a specific site, i.e., the radioactivity which can not be competed for by excess ligand. However, this excess ligand may interfere with, for example, pinocytotic activity or with the mor-

phology of the cell, because of which the nonspecific binding at nanomolar or millimolar concentrations of ligand may be different. Therefore, we have been cautious in accepting the reality of the very slowly dissociating binding component. However, nonspecific binding is constant with time [5], and this binding type is observed if dissociation of bound [^3H]cAMP is followed after the addition of excess unlabelled cAMP or after an 11-fold dilution of the incubation mixture (unpublished observations). Furthermore, this binding type is not only observed in cells at 0°C (Fig. 4), but also in cells at 20°C or in membranes prepared by shearing the cells through Nucleopore filters (unpublished observations).

The dissociation of 2 nM [^3H]cAMP from the cells in buffer at 0°C is shown in Fig. 4A. Cells were preincubated in 0.1 ml with 2 nM [^3H]cAMP for 1 min. Then at $t = 0$, 1 ml buffer with 10^{-4} M cAMP is added, the suspension is shaken and 1 ml is centrifuged through silicon oil at the times indicated. With this procedure nonspecific binding is very low (10.8 ± 1.0 cpm ($n = 5$) with an input of 7600 cpm). Total binding at 10 min after the chase with cAMP is 27.9 ± 1.4 cpm ($n = 4$), which is significantly above nonspecific binding. The off-rate of the very slow component is obtained from the slope of the data between 4 and 10 min after the cAMP chase ($k_{-1} = 1.6 \cdot 10^{-3} \text{ s}^{-1}$, $t_{1/2} = 7.2$ min). Assuming that the off-rate is constant between 0 and 10 min, about 10% of the radioactivity was bound to this component (intercept with ordinate). Binding to this component is calculated by the equation

$$b^{ss}(t) = 0.1 b(o) e^{-k_{-1}t}$$

where $b^{ss}(t)$ is the radioactivity bound to the very slow component, and k_{-1} is the rate constant of dissociation of this component. Subtraction of $b^{ss}(t)$ from the observed binding $b(t)$ and a replot of the result as $\ln(b(t) - b^{ss}(t)) / (b(o) - b^{ss}(o))$ vs. time still yields a curved line with a slow component (18% with $k_{-1} = 1.6 \cdot 10^{-2} \text{ s}^{-1}$) and a fast component (72% with $k_{-1} \approx 0.2 \text{ s}^{-1}$).

The effect of ammonium sulfate on the dissociation of the cAMP receptor complex is shown in Fig. 4B. Cells were incubated with 2 nM [^3H]cAMP in 3.4 M ammonium sulfate for 5 min. Then excess

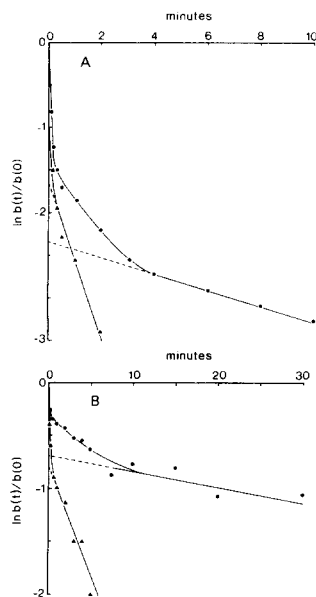


Fig. 4. Dissociation of the cAMP receptor complex. A. Dissociation in buffer. 10^7 cells were preincubated for 1 min in 0.1 ml assay buffer with 2 nM [3 H]cAMP (7600 cpm). At $t = 0$ min 1 ml 0.1 nM cAMP in assay buffer was added, and 1 ml of the mixture was centrifuged through silicon oil at the times indicated. Binding at the onset of the cAMP chase was determined by centrifugation of 91 μ l of the preincubation mixture through silicon oil. Nonspecific binding was determined by including 0.1 mM cAMP in the preincubation mixture, and was 42 cpm before the cAMP chase ($t = 0$) and 11 cpm after the cAMP chase (the difference is due to the 11-fold dilution of the preincubation mixture). $b(0)$ is the specific binding at the onset of the cAMP chase (450 cpm), at $b(t)$ is the specific binding at t min after the chase. The data (\bullet) between 4 and 10 min indicate that about 10% of the radioactivity dissociates with a $k_{-1} = 1.6 \cdot 10^{-3} \text{ s}^{-1}$. The residual binding to this component between 0–2 min was subtracted from observed binding, and the result was replotted (Δ). This indicates that about 18% of the radioactivity bound at $t = 0$ dissociates with $k_{-1} = 1.6 \cdot 10^{-2} \text{ s}^{-1}$ and about 72% with $k_{-1} \approx 0.2 \text{ s}^{-1}$. B. Dissociation in ammonium sulfate. 10^7 cells were preincubated for 5 min in 1 ml assay buffer containing 2 nM [3 H]cAMP, 3.4 M ammonium sulfate and 0.5 mg bovine serum albumin. At $t = 0$ min 10 μ l 10 mM cAMP were added, and samples were centrifuged at the times indicated. The observed specific binding after the cAMP chase (\bullet) was resolved in to three components, as in A. This indicates that 50% of the radioactivity bound at $t = 0$ dissociates with $k_{-1} = 2.5 \cdot 10^{-4} \text{ s}^{-1}$, 23% with $k_{-1} = 3.5 \cdot 10^{-3} \text{ s}^{-1}$ and 27% with $k_{-1} \approx 7 \cdot 10^{-2} \text{ s}^{-1}$.

cAMP was added, and specific binding was determined. Also in the presence of 3.4 M ammonium sulfate the dissociation can be resolved in

three components; however, the off-rates and the proportioning of the components are different. Approx. 50% of the radioactivity dissociates in 3.4 M ammonium sulfate with a half-life of about 46 min ($k_{-1} = 2.5 \cdot 10^{-4} \text{ s}^{-1}$), approx. 23% with a half-life of about 200 s ($k_{-1} = 3.5 \cdot 10^{-3} \text{ s}^{-1}$), and about 27% with a half-life of about 10 s ($k_{-1} = 7 \cdot 10^{-2} \text{ s}^{-1}$). It has not been proven that the three components observed in buffer are the same binding types as those three observed in ammonium sulfate, especially because a still slower component may exist in ammonium sulfate, since only 70% of the cAMP receptor complexes have dissociated during the time of the experiment. Nevertheless, it is obvious that the cAMP receptor complex can dissociate in 3.4 M ammonium sulfate, and that dissociation is at least 3–5-fold slower than in buffer. Additionally, ammonium sulfate shifts the proportioning of the cAMP-binding components to the types with the slower dissociation rate. Thus, in buffer 75% of the [3 H]cAMP bound has dissociated in about 20 s, while the same fraction has dissociated in ammonium sulfate only after 30 min, a 90-fold difference.

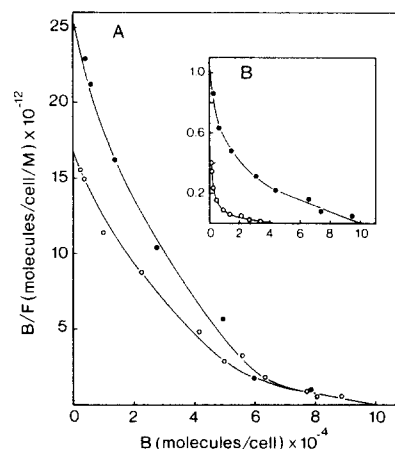


Fig. 5. The effect of CaCl_2 on cAMP-binding in the presence (A) or absence (B) of 3.4 M ammonium sulfate. A. The incubation mixture contained, in 1 ml assay buffer, 10^7 cells, 3.4 M ammonium sulfate, 0.5 mg bovine serum albumin, different concentrations of cAMP in the absence (\circ) or presence (\bullet) of 10 mM CaCl_2 . Samples were centrifuged after 5 min. B. The incubation mixture contained, in 0.1 ml assay buffer, 10^7 cells, different concentrations of cAMP with (\bullet) or without (\circ) 10 mM CaCl_2 . Samples (95 μ l) were centrifuged through silicon oil after 1 min.

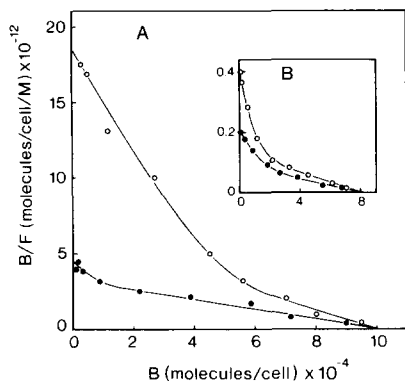


Fig. 6. The effect of caffeine on cAMP-binding in the presence (A) or absence (B) of 3.4 M ammonium sulfate. The experiment was identical to the one presented in Fig. 5, except that CaCl_2 was replaced by 10 mM caffeine. \circ , without caffeine; \bullet , with caffeine.

Effect of Ca^{2+} and caffeine on cAMP-binding in ammonium sulfate

Juliani and Klein [11] have shown that millimolar concentrations of Ca^{2+} induce an approx. 2–3-fold increase in the number of cAMP-binding sites. Approximately the same increase is induced by 50 mM ammonium sulfate (Fig. 2). Therefore, the number of cAMP-binding sites was determined in ammonium sulfate in the absence or presence of 10 mM CaCl_2 (Fig. 5). This demonstrates that CaCl_2 and ammonium sulfate induce the same number of binding sites, and that the increase in cAMP-binding by ammonium sulfate is not further enhanced by CaCl_2 .

Caffeine has been shown to inhibit cAMP-binding by reducing the apparent affinity without an effect on the number of cAMP-binding sites (Fig. 6) [5]. The same observation is made if cAMP-binding is measured in the presence of 3.4 M ammonium sulfate, except that the reduction of apparent affinity is more pronounced in ammonium sulfate than in buffer.

Discussion

At the introduction of the ammonium sulfate stabilization assay [3] we mentioned some preliminary experiments which suggested that the association of cAMP to the receptor and the dissociation of the cAMP receptor complex do not take

place in the presence of about 3.5 M ammonium sulfate. The more detailed experiments in the present report clearly indicate that association and dissociation are not prevented by ammonium sulfate. Previously the cells were incubated in 0.1 ml with $[^3\text{H}]\text{cAMP}$ for 1 min, which was followed by a 12-fold dilution with ammonium sulfate. Due to our view that ammonium sulfate ‘freezes’ the existing equilibrium as in other receptors [6,7], we assumed that specific binding in the cell pellet was identical to the binding of cAMP before the addition of ammonium sulfate. The expression of cAMP bound as pmol bound/mg protein or as molecules/cell remains identical whether it is calculated for the situation before or after the addition of ammonium sulfate. However, the expression of cAMP bound as nM should be 12-fold lower after the addition of ammonium sulfate. Therefore, all data reported previously [2,3,12] remain identical, except that the apparent K_d values obtained in Scatchard plots are 12-fold lower. In the present report we show that ammonium sulfate induces an approx. 15-fold reduction in K_d ; therefore the K_d values reported previously are probably very similar to those measured in the absence of ammonium sulfate.

It is evident from the present results that ammonium sulfate has a multiple effect on cell surface cAMP receptors. Firstly, at low ammonium sulfate concentrations (about 50 mM) the number of cAMP-binding sites increased about 2-fold. The same increase is observed with millimolar concentrations of Ca^{2+} ; Ca^{2+} and ammonium sulfate are not additive. A similar observation has been made recently by Janssens and Van Driel [13], who presented evidence that this increase is a nonspecific effect induced by many salts.

Secondly, at high ammonium sulfate concentrations (up to 3.5 M) a 15-fold increase in the affinity is observed. This increase in affinity is partly due to an alteration of the cAMP-binding heterogeneity. *D. discoideum* cells contain fast-dissociating binding sites with relatively low affinity ($t_{1/2} \approx 1$ s; $K_d \approx 60$ –450 nM), and slowly dissociating binding sites with high affinity ($t_{1/2} = 15$ –150 s; $K_d \approx 15$ nM; all data measured at 20°C; Ref. 5 and unpublished results). The distribution of cAMP-binding at 2 nM $[^3\text{H}]\text{cAMP}$ is shifted by ammonium sulfate to the slowly dissociating bind-

ing components with high affinity. Caffeine has been shown to alter high-affinity binding to low-affinity binding [5]. Caffeine inhibits cAMP-binding more strongly in the presence of ammonium sulfate than in its absence, because ammonium sulfate enhances the high-affinity components.

Finally, high concentrations of ammonium sulfate increase the affinity of all cAMP-binding components because the dissociation rates are reduced about 3–5-fold. Thus the 30-fold increase in cAMP-binding at 1 nM [^3H]cAMP by 3.5 nM ammonium sulfate is composed of a 2-fold increase in the cAMP-binding sites, a 3–6-fold increase in the affinity of all binding components, and an alteration in the binding heterogeneity to the components with the higher affinity, which contributes to an approx. 4-fold increase in cAMP-binding.

The effect of ammonium sulfate on the reduced dissociation of the ligand receptor complex has been observed in other receptors [6–7]. The present results suggest that this effect is not due to a blockade of the mobility of the ligand to, in or from the binding site, because association and dissociation of the ligand to the receptor are still possible. Although we have presented details on the mode of action of ammonium sulfate, its mechanism of action is still largely unknown.

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